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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 93/05396
G01N 33/566, 33/68, C12Q 1/00	A1	(43) International Publication Date: 18 March 1993 (18.03.93)

(21) International Application Number: PCT/DK92/00270

(22) International Filing Date: 9 September 1992 (09.09.92)

(30) Priority data:
PCT/DK91/00264 12 September 1991 (12.09.91) WO
(34) Countries for which the regional or international application was filed: DK et al.

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(81) Designated States: AU, CA, CS, FI, HU, JP, PL, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: A METHOD OF SCREENING FOR INHIBITORS OF HEPARIN-BINDING PROTEIN

(57) Abstract

Inhibitors of heparin-binding protein are screened for by incubating HBP or a cell producing HBP with a substance suspected of being an HBP inhibitor and with tissue, cells or a component thereof capable of interacting with HBP, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or component thereof, decreased interaction indicating that said substance is an HBP inhibitor.

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A METHOD OF SCREENING FOR INHIBITORS OF HEPARIN-BINDING PROTEIN

FIELD OF INVENTION

The present invention relates to methods of screening for substances which are inhibitors or antagonists of heparin-binding protein, as well as to a test kit for use in the method.

BACKGROUND OF THE INVENTION

The covalent structure of two closely related proteins isolated from peripheral neutrophil leukocytes of human and porcine origin have recently been determined (cf. H. Flodgaard et al., Eur. J. Biochem. 197, 1991, pp. 535-547; J. Pohl et al., FEBS Lett. 272, 1990, p. 200 ff.). Both proteins show a high similarity to neutrophil elastase, but owing to selective mutations of the active serine 195 and histidine 57 (chymotrypsin numbering (B.S. Hartley, "Homologies in Serine Proteinases", Phil. Trans. Roy. Soc. Series 257, 1970, p. 77 ff.)) the proteins lack protease activity. The proteins have been named human heparin-binding protein (hHBP) and porcine heparin-binding protein (pHBP), respectively, owing to their high affinity for heparin; Schafer et al. (W.M. Schafer et al., Infect. Immun. 53, 1986, p. 651 ff.) have named the protein cationic antimicrobial protein (CAP37) due to its antimicrobial activity. The protein has also been shown to be chemotactic for monocytes over the range 1.3 x 10^{-9} M - 10^{-8} M (H.A. Pereira et al., J. Clin.Invest. 85, 1990, p.1468 ff.), consistent with the results apparent from Flodgaard et al., op. cit..

Furthermore, HBP has been shown to mediate detachment and contraction of endothelial cells and fibroblasts when added to such cells grown in monolayer culture. HBP also stimulates monocyte survival and thrombospondin secretion (E. Østergaard and H. Flodgaard, J. Leukocyte Biol. 51, 1992, p 316 ff.

From the azurophil granules, a protein with the first 20 N-terminal amino acid residues identical to those of hHBP and CAP37 called azurocidin has also been isolated (J.E. Gabay et al., Proc. Natl. Acad. Sci. USA 86, 1989, p. 5610 ff.; C.G. Wilde et al., J. Biol. Chem. 265, 1990, p. 2038 ff.) and its antimicrobial properties have been reported (D. Campanelli et al., J. Clin. Invest. 85, 1990, p. 904 ff.).

The presence of hHBP in the neutrophil leucocytes and the fact that 89% of CAP37 (which is identical to hHBP) is released when the leucocytes are phagocytosing Staph. aureus (H.A. Pereira et al., op. cit.) indicate that a function of hHBP could be its involvement in the inflammatory process since the protein is apparently released from activated neutrophils. Pereira et al., op. cit., suggested a function of CAP37 to be at the site of inflammation where it could specifically attract monocytes and thus be one of the factors responsible for the influx of monocytes in the second wave of inflammation. Østergaard and Flodgaard, op. cit., suggest that, in addition to being important for the recruitment of monocytes, HBP might play a key role in the mechanism of neutrophil as well as monocyte extravasation.

SUMMARY OF THE INVENTION

The observation that heparin-binding protein induces morphological changes in fibroblast and endothelial cell monolayers with marked cell contraction and disruption of the cellular monolayer as well as chemotaxis of monocytes suggests that heparin-binding protein plays an important part in the migration of polymorphonuclear leukocytes (PMNs) to an inflammatory site through the vascular wall. The inhibition of heparin-binding protein may therefore be desirable in cases where the immune response occurs in an exaggerated or inappropriate form, as for instance in various types of hypersensitivity, especially the classical types I, II and III. As these types of hypersensitivity are antibody-mediated,

complement activation occurs and PMNs are attracted to the site f deposition f immune complexes, thereby causing local damage. Inhibition of heparin-binding protein may therefore suppress extravasation of PMNs in immune complex mediated conditions such as vasculitis, nephritis, rheumatoid arthritis, asthma, acute lung distress syndrome and chronic irritation of lung tissue.

It is also well known that in the chronic state of these inflammatory diseases, mononuclear phagocytes constitute a prominent part of the infiltrate, and studies of the synovium in rheumatoid arthritis show that the increase in macrophages is due to an increase in the influx of blood monocytes (N. Hogg et al., Immunology 56, 1985, pp. 673-681). This phenomenon is believed to occur through chemotactic migration towards the focus of inflammation. Interference with this process might be achieved by inhibition of heparin-binding protein mediated monocyte chemotaxis as well as inhibition of heparin-binding protein mediated differentiation of monocytes to macrophages.

Other indications in which a beneficial effect of an inhibition of heparin-binding protein might be obtained are hyperacute graft rejection, post-ischemic myocardial injury and severe burns. In these cases, heavy infiltration by neutrophils causes the damage observed.

The present invention relates to a screening method for inhibitors of heparin-binding protein.

Accordingly, in one aspect, the invention relates to a method of screening for an inhibitor of heparin-binding protein (HBP), the method comprising incubating HBP or a cell producing HBP with a substance suspected of being a HBP inhibitor, and subsequently with tissue, cells or a component thereof capable of interacting with HBP, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or compon nt thereof, decreased interaction indicating that said

substance is a HBP inhibitor.

In another aspect, the invention relates to a method of screening for an inhibitor of HBP, the method comprising incubating HBP or a cell producing HBP with tissue, cells or a component thereof capable of interacting with HBP, and subsequently with a substance suspected of being a HBP inhibitor, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or component thereof, decreased interaction indicating that said substance is a HBP inhibitor.

In a further aspect, the invention relates to a method of screening for an inhibitor of HBP, the method comprising incubating a substance suspected of being a HBP inhibitor with tissue, cells or a component thereof capable of interacting with HBP, and subsequently with HBP or a cell producing HBP, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or component thereof, decreased interaction indicating that said substance is a HBP inhibitor.

In the present context, the term "heparin-binding protein" ("HBP") is intended to indicate a homologue of neutrophil elastase without proteolytic activity. Based on in vitro data, it is currently believed that HBP may be involved in the diapedesis of neutrophils and assist their migration by inducing contraction of fibroblast, thus facilitating elastase-mediated digestion of barrier extracellular matrix proteins. HBP continuously secreted from migrating neutrophils may then form a haptotactic gradient which attracts peripheral monocytes to the injured tissue in the "second wave" of inflammation. The structure of HBP appears from WO 89/08666 and H. Flodgaard et al., op. cit. HBP has otherwise been termed CAP37 (cf. WO 91/00907) and azurocidin (cf. C.G. Wilde et al., J. Biol. Chem. 265, 1990, p. 2038). The HBP may suitably be of mammalian, in particular human, origin. The term is intended to include functional analogues, i.e. polypeptides which have

a similar function as the native protein. Examples of such functional analogues include derivatives of the native protein obtained by addition of one or more amino acid residues to either or both the C- or N-terminal end of the native protein, substitution of one or more amino acid residues at either or both ends of the native protein, deletion of one or more amino acid residues at either or both ends of the native protein or at one or more sites within the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the native amino acid sequence. The term is specifically intended to include peptide fragments of HBP, in particular fragments with a similar chemotactic effect as native HBP.

In the course of an inflammatory injury, HBP is believed to be secreted from neutrophils which adhere to the endothelium adjacent to the injury. The released HBP present in the microenvironment between the adhering neutrophils and the endothelial cells has been found to bind to a receptor present on the endothelial cells, which is currently assumed to be an integrin receptor.

Integrins which are widely recognized as the major receptors by which cells adhere to extracellular matrices and, for some integrins, to other cells are expressed on a variety of cells, including endothelial cells. Integrins are composed of α and β subunits, wherein the α subunits contain sequences of predominantly negatively charged amino acids (Asp-x-Asp-x-Asp-Gly-x-x-Asp, or the like) which are believed make the α subunits able to bind divalent cations such as Ca^{2+}, Mn^{2+} or Mg^{2+} (more information on integrins in general is found in R.O. Hynes, Cell 69, 1992, pp. 11-25). The presence of divalent cations on the integrin receptors is essential for their ligand binding ability. For instance a mutation introduced in the integrin $\alpha_{IIb}\beta_3$ causes defective divalent cation binding and consequently loss of ligand binding ability (J.C. Loftus et al., Science 249, 1990, pp. 915-918.

It has now been found that HBP binds to negatively charged sequences of the integrin receptor by a positively charged sequence (e.g. Arg-Arg-Arg-Glu-Arg-Glu-Ser-Arg in positions 74-80 of human HBP), thus competing for binding of divalent cations to the receptor, and consequently the integrin is turned to an "off" conformation. This process is thought to result in a limited detachment from the basement membrane followed by a limited contraction of the cells. This sequence of phenomena may facilitate neutrophil diapedesis between adjacent endothelial cells and eventually through the basement membrane. The same mechanism is thought to be involved in neutrophil migration through all types of barrier cell layers through which the neutrophils have to pass when challenged by an inflammatory signal. Such layers may, for instance, be composed of fibroblasts, smooth muscle cells, mesothelial cells, alveolar epithelial cells, intestinal epithelial cells and serosal cells.

In the present context, the term "inhibitor" is used to indicate a substance which inhibits HBP binding to endothelial cells, fibroblasts, smooth muscle cells and monocytes by competing with HBP for binding to a receptor on such cells. This substance should preferably be one capable of binding to the receptor with an affinity which is at least as high as that of HBP itself, and it should be a substance which, unlike HBP, is unable to mediate any signal through the receptor. If the receptor is indeed an integrin, as indicated above, the inhibitor may preferably be a substance which is either capable of binding to the receptor binding site of HBP, thereby preventing HBP from binding to its receptor, or capable of binding to HBP at a site at which it caps or covers the receptor binding site such that HBP binding to its receptor is sterically hindered.

In a still further aspect, the present invention relates to a test kit for screening for a HBP inhibitor, the kit comprising, in s parate containers,

(a) HBP or a cell producing HBP, and

(b) tissue, cells or a component thereof capable of interacting with HBP.

DETAILED DESCRIPTION OF THE INVENTION

According to the invention, the tissue, cells or component thereof capable of interacting with HBP may be selected from the group consisting of (a) endothelial cells, fibroblasts or smooth muscle cells or a component thereof, (b) connective tissue or a component thereof, or (c) monocytes or a component thereof. The interaction of such cells with HBP is evidenced by their contraction (fibroblasts and endothelial cells) or aggregation (monocytes) in the presence of HBP, as described in further detail below.

Thus, in one embodiment of the method of the invention, the HBP or HBP-producing cell may be incubated with fibroblasts, endothelial cells or smooth muscle cells present in a confluent layer on a solid support, or with monocytes in suspension. By adding the suspected HBP inhibitor, any decreased degree of cell contraction or aggregation, or restoration of the confluent layer of cells indicates the presence of a HBP inhibitor. Conversely, a suspected HBP inhibitor or antagonist may be incubated with fibroblasts, endothelial cells or smooth muscle cells present in a confluent layer on the solid support, or monocytes in suspension, after which HBP or a HBP-producing cell is added. Any decrease in or prevention of cell contraction or aggregation indicates the presence of a HBP inhibitor.

The solid support on which the cells are grown to confluence may be any conventional material usually employed for this purpose, e.g. a plastic such as latex, polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylalcohol, nylon, polyvinylacetate, or any suitable

copolymer thereof, or it may be a polymer component of connective tissue such as collagen, gelatin or fibronectin. A convenient shape of the solid support is a culture dish. Alternatively, when the cells with which the HBP or suspected HBP inhibitor is incubated are monocytes, the HBP may be immobilised on a solid support.

When the HBP or HBP-producing cell, or the suspected HBP inhibitor is incubated with a component of endothelial cells, fibroblasts or smooth muscle cells, said component preferably comprises a surface membrane fraction or molecule, such as a HBP receptor. In this embodiment of the assay, the HBP may be immobilized on a solid support. Alternatively, the HBP may be provided with a suitable label.

The solid support employed in the screening method of the invention preferably comprises a polymer. The support may in itself be composed of the polymer or may be composed of a matrix coated with the polymer. The matrix may be of any suitable material such as glass, paper or plastic. The polymer may be selected from the group consisting of a plastic (e.g. latex, a polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylalcohol, nylon, polyvinylacetate, and any suitable copolymer thereof), cellulose (e.g. various types of paper, such as nitrocellulose paper and the like), a silicon polymer (e.g. siloxane), a polysaccharide (e.g. agarose or dextran), an ion exchange resin (e.g. conventional anion or cation exchange resins), a polypeptide such as polylysine, or a ceramic material such as glass (e.g. controlled pore glass).

The physical shape of the solid support is not critical, although some shapes may be more convenient than others for the present purpose. Thus, the solid support may be in the shape of a plate, e.g. a thin layer or microtiter plate, or a film, strip, membrane (e.g. a nylon membrane or a cellulose filter) or solid particles (e.g. latex beads or dextran or agarose beads).

The label substanc with which the HBP may be labelled is preferably sel cted from the group consisting of enzymes, coloured or fluorescent substances, radioactive isotopes and complexing agents.

Examples of enzymes useful as label substances are peroxidases (such as horseradish peroxidase), phosphatases (such as acid or alkaline phosphatase), ß-galactosidase, urease, glucose oxidase, carbonic anhydrase, acetylcholinesterase, glucoamylase, lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase, ß-glucosidase, proteases, pyruvate decarboxylase, esterases, luciferase, etc.

Enzymes are not in themselves detectable but must be combined with a substrate to catalyse a reaction the end product of which is detectable. Examples of substrates which may be employed in the method according to the invention include hydrogen peroxide/tetramethylbenzidine or chloronaphthole or o-phenylenediamine or 3-(p-hydroxyphenyl) propionic acid or luminol, indoxyl phosphate, p-nitrophenylphosphate, nitrophenyl galactose, 4-methyl umbelliferyl-D-galactopyranoside, or luciferin.

Alternatively, the label substance may comprise coloured or fluorescent substances, including gold particles, coloured or fluorescent latex particles, dye particles, fluorescein, phycoerythrin or phycocyanin.

Radioactive isotopes which may be used for the present purpose may be selected from I-125, I-131, In-111, H-3, P-32, C-14 or S-35. The radioactivity emitted by these isotopes may be measured in a gamma-counter or a scintillation camera in a manner known per se.

Complexing agents which may be employed for the present purpose may be selected from biotin (which complexes with avidin or streptavidin), avidin (which complexes with bi tin), Protein

A (which complexes with immunoglobulins) and lectins (complexing with carbohydrate receptors). As the complex is not directly detectable, it is necessary to label the substance with which the complexing agent forms a complex. The labelling may be carried out with any one of the label substances mentioned above for the labelling of the enzyme.

The HBP receptor which may be used in the screening method of the invention, may be used in isolated form and may, as such, be provided with a label or may be immobilised on a solid support, respectively, as described above. However, the receptor may also be used in membrane-bound form, i.e. bound to whole cells or as a component of membrane preparations. If the receptor is bound to whole cells (expressed on their surface), binding of the receptor to HBP may be measured by counting the cells visually, or by measuring naturally occurring intracellular enzyme activity, e.g. cathepsin B activity, or by measuring an enzyme activity introduced into the cells by recombinant DNA techniques.

More particularly, an assay for HBP inhibitors may be established by incubating the HBP receptor immobilised on a solid support or cells expressing a HBP receptor or a surface membrane fraction thereof, likewise immobilised on a solid support, with labelled HBP and a suspected HBP inhibitor and measuring the amount of HBP bound to the receptor. Decreased binding of HBP (compared to a control which has not been incubated with the suspected inhibitor) indicates an inhibitory effect of the test substance in question.

It is further envisaged that an antibody reactive with HBP may be added after incubation of (unlabelled) HBP or cells producing HBP, the substance suspected of being a HBP inhibitor and tissue, cells or a component thereof capable of interacting with HBP. In this cas , the antibody may be provided with a label as indicated above, or a labelled second antibody reactive with the anti-HBP antibody may be added after addition

of the anti-HBP antibody.

Based on current knowledge of HBP binding to the integrin receptor as indicated above, possible inhibitors of HBP binding are expected to be substances with an overall negative net charge. Examples of such substances are peptides containing one or more Asp and/or Glu residues, or sulfated carbohydrates such as dextran sulfate, heparan sulfate or sucralfate.

The HBP used in the present screening method is preferably in substantially pure form in order to avoid possible interference from other substances present in the assay. The HBP is therefore most conveniently prepared by recombinant DNA techniques, for instance as follows.

A DNA sequence encoding HBP may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA sequence may also be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of HBP by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989). The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

The DNA sequence is then inserted into a recombinant xpression vector which may be any vector which may conveniently be

subjected to recombinant DNA procedures. The choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding HBP should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding HBP in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter.

The DNA sequence encoding HBP may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise

elements such as polyadenylation signals (e.g. fr m SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate.

The procedures used to ligate the DNA sequences coding for HBP, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The host cell into which the expression vector is introduced may be any cell which is capable of producing HBP and is preferably a eukaryotic cell, in particular a mammalian cell. Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells (including yeast cells) may be used

as host cells. Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of Saccharomyces cerevisiae. Examples of other fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 238 023.

The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The HBP produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

The invention is described in further detail in the following example which is not in any way intended to limit the scope of the invention as claimed.

EXAMPLE

Materials

Tissue culture trays were purchased from Nunc, and fresh "buffy coats" were obtained from the blood bank. Ficoll-Paque, Percoll and Protein A-Sepharose CL-4B were obtained from Pharmacia. MRC-5 embryo lung fibroblasts and fetal bovine heart endothelial (FBHE) cells were obtained from the American Type

Culture Collection. The myeloid leukemic cell line U937 (cf. C. Sundström and K. Nilsson, Int. J. Cancer 17, 1976, pp. 565-577) was obtained from A. Fattorsi, Research Laboratories of Aeronautica Militare, Rome, Italy. Human HBP (hHBP) (more than 95 % pure as judged by polyacrylamide gel analysis) was purified from neutrophil leucocytes as described in H. Flodgaard et al., op. cit. Phorbol 12 myristate 13 A (PMA) was obtained from Sigma. Na-sucrose-oktakis-(hydrogen sulfate)aluminium complex (sucralfate) was obtained from Bukh Meditec A/S, Farum, Denmark.

Isolation of monocytes

Mononuclear cells were isolated from healthy donors, essentially according to the method described by Böyum (J. Clin. Lab. Invest. 21 (suppl. 97), 1968, p. 77). Briefly, "buffy coats" were diluted with 1 volume of cold Dulbecco's modified Eagle medium (DME) and layered on the top of 15 ml of Ficoll-Paque in 50 ml Falcon tubes. After centrifugation at 400 x g for 30 min. in a swingout rotor, the layer between Ficoll-Paque and DME-plasma (containing the mononuclear cells and blood platelets) was collected, and the platelets were subsequently removed by repeated washing of the cells in DME. The mononuclear cells were fractionated further by centrifugation on a Percoll gradient (generated by centrifugation of isotonic Percoll with an average density of 1,070 g/ml at 3.200 x g for 15 min. in a fixed angle rotor (J.C. Giddings et al., Clin.Lab. Haemat. 2, 1980, p.121)). The mononuclear cells were layered at the top of the gradient and centrifuged at 2.700 x g for 20 min. in a swing out rotor. The monocytes at the top of the gradient were determined by nonspecific esterase staining to be of a purity in excess of 90%. When monocytes were incubated with hHBP, the cells were initially plated in DME supplemented with 10 mg/ml BSA prior to addition of hHBP. The monocytes were incubated in DME (with less than 25 pg of endotoxin per ml medium) supplemented with penicillin and streptomycin. The cultures were photographed aft r 16 h of incubation with an Olympus OM-4 camera adapted to an Olympus

CK-2 inverted microscope.

Morphological studies on human MRC-5 embryo lung fibroblasts and FBHE treated with hHBP.

MRC-5 cells were used between passage 20 and 35 and cultured in minimal essential medium (MEM) with 1% L-glutamine, 1.1% $NaHCO_3$, 1% non-essential amino acids, penicillin/streptomycin and 10% FCS. FBHE cells were cultured in DME containing 10% newborn calf serum (NCS) and 10-20 ng/ml recombinant human basic fibroblast growth factor. Both cell types were grown to confluence in 24- well macrowell dishes and washed once in DME before treatment with hHBP. The cells were incubated with hHBP in DME with penicillin/streptomycin for 16 h at 37°C in a humidified atmosphere containing 5 % CO_2. They were then photographed.

Changes in cellular morphology after incubation with hHBP

When confluent MRC-5 or FBHE cells were incubated with 10 µg/ml of hHBP added to the culture medium, cell contraction could be observed after 16 hours of incubation, leaving extensive gaps between cells. Control fibroblasts were confluent, long thin and spindle shaped due to lack of serum, and control endothelial cells were typical flat cobblestone-like cells. Monocytes incubated with 10 µg/ml of hHBP for 16 hours aggregated into large multicellular clumps, whereas control monocytes adhered firmly to the surface of the well. Morphological changes of the monocytes could be observed within 2 h as cells showed a more rounded morphology, but cellular aggregation only appeared several hours later. Clump formation of MRC-5 fibroblasts and FBHE cells was also observed if the cells were seeded in DME without added serum in the presence of hHBP at a concentration of 10 µg/ml. At higher concentrations of hHBP (30 µg/ml), monolayers of MRC-5 and FBHE detached from the surface of the well and formed aggregates. The contraction of MRC-5 fibroblasts and FBHE cells was reversible when concentrations of hHBP up t 10 µg/ml was used, as addition of foetal calf serum to 10 % completely restored

the confluent monolayers 24 hours later. At higher concentrations of hHBP, the cells were trapped in aggregates and were therefore unable to move to the substrate after addition of foetal calf serum.

Homotypic aggregation of U937 cells

The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were washed twice in DMEM without FCS and seeded to a density of $0.5x10^6$ cells/ml with 150 nM PMA in DMEM in 24-well macrowell dishes. 10 μg of HBP were added to the test wells and PBS to control wells followed by incubation at 37°C in a humidified atmosphere containing 5% CO_2. The culture were inspected occasionally and photographed after 16 hours of incubation.

Homotypic aggregation could be recognized after 2 hours, culminating after 12-16 hours. In the controls, the cells adhered to the bottom of the wells and were well spread.

In their unstimulated condition, U937 cells grow in suspension independently of anchorage. On PMA stimulation, however, this cell line shows changes in the cell surface expression of the β_2 integrins CD11b/CD18 and CD11c/CD18. A significant increase in the expression of these integrins and the loss of CD71 characterizes the differentiation of U937 cells into macrophage-like cells (cf. C. Cabañas et al., Hybridoma 7, 1988, pp. 167-176).

The strong homotypic aggregation observed on the addition of HBP to PMA-stimulated U937 cells, indicates that HBP affects the integrins (probably in the divalent ion binding site) and mediates homotypic aggregation in a similar way as reported for a monoclonal antibody reactive with this epitope (cf. D.C. Altieri, J. Biol. Chem. 147, 1991, pp. 1891-1898; and C. Cabañas et al., Biochim. Biophys. Acta 1092, 1991, pp. 165-168.

This experimental set-up may be used to screen for HBP inhibitors by adding a test substance suspected of being a HBP inhibitor before or after adding HBP, and determining any effect of the test substance on the contraction of fibroblasts or endothelial cells or on the aggregation of monocytes described above.

Thus, in a similar set-up, the effect of Na-sucrose-oktakis-(hydrogen sulfate) aluminium complex (sucralfate) was tested. The addition of sucralfate to a concentration of 100 µg/ml of medium to control wells not containing any HBP did not influence the PMA-stimulated adherence and spreading of the cells. However, when HBP to a concentration of 10 µg/ml of medium was added to the wells, the same concentration of sucralfate strongly suppressed the homotypic aggregation of the cells described above. The mechanism behind the inhibition of HBP is believed to be caused by an electrostatic neutralization of the Arg-Arg-Arg-Glu-Arg-Glu-Ser-Arg motif in positions 74-80 of HBP by the strongly negatively charged sucralfate molecule.

CLAIMS

1. A method of screening for an inhibitor of heparin-binding protein (HBP), the method comprising incubating HBP or a cell producing HBP with a substance suspected of being a HBP inhibitor, and subsequently with tissue, cells or a component thereof capable of interacting with HBP, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or component thereof, decreased interaction indicating that said substance is a HBP inhibitor.

2. A method of screening for an inhibitor of heparin-binding protein (HBP), the method comprising incubating HBP or a cell producing HBP with tissue, cells or a component thereof capable of interacting with HBP, and subsequently with a substance suspected of being a HBP inhibitor, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or component thereof, decreased interaction indicating that said substance is a HBP inhibitor.

3. A method of screening for an inhibitor of heparin-binding protein (HBP), the method comprising incubating a substance suspected of being a HBP inhibitor with tissue, cells or a component thereof capable of interacting with HBP, and subsequently with HBP or a cell producing HBP, and detecting any effect of said substance on the interaction of HBP with said tissue, cells or component thereof, decreased interaction indicating that said substance is a HBP inhibitor.

4. A method according to claim 1, 2 or 3, wherein the tissue, cells or component thereof capable of interacting with HBP is selected from the group consisting of (a) endothelial cells, fibroblasts or smooth muscle cells or a component thereof, (b) connective tissue or a component thereof, or (c) monocytes or a component thereof.

5. A method according to claim 4, wherein, when the HBP or HBP-

producing cell, or the suspected HBP inhibit r is incubated with end thelial cell, fibroblasts or smooth muscle cells, the endothelial cells, fibroblasts or smooth muscle cells are present in a confluent layer on a solid support.

6. A method according to claim 5, wherein the solid support comprises a polymer such as collagen, gelatin or fibronectin, or a plastic, e.g. latex, polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylalcohol, nylon, polyvinylacetate, or any suitable copolymer thereof.

7. A method according to claim 4, wherein, when the HBP or HBP-producing cell, or the suspected HBP inhibitor is incubated with a component of endothelial cells, fibroblasts or smooth muscle cells, said component comprises a surface membrane fraction or molecule.

8. A method according to claim 7, wherein the surface membrane molecule is a HBP receptor.

9. A method according to claim 4, wherein, when HBP is incubated with monocytes, the HBP is immobilised on a solid support.

10. A method according to any of claims 1-9, wherein the HBP is recombinant HBP.

11. A method according to any of claims 1-10, wherein the HBP is provided with a label.

12. A method according to any of claims 1-11, wherein an antibody reactive with HBP is added after incubation of HBP or cells producing HBP, the substance suspected of being a HBP inhibitor and tissue, cells or a component thereof capable of interacting with HBP.

13. A method according to claim 12, wherein the antibody is

provided with a label.

14. A method according to claim 12, wherein a labelled second antibody reactive with the anti-HBP antibody is added after addition of the anti-HBP antibody.

15. A test kit for screening for a HBP inhibitor or antagonist, the kit comprising, in separate containers,

(a) HBP or a cell producing HBP, and

(b) tissue, cells or a component thereof capable of interacting with HBP.

16. A test kit according to claim 15, wherein the tissue, cells or component thereof capable of interacting with HBP is selected from the group consisting of (i) endothelial cells, fibroblasts or smooth muscle cells or a component thereof, (ii) connective tissue or a component thereof, or (iii) monocytes.

17. A test kit according to claim 16, wherein the endothelial cells, fibroblasts or smooth muscle cells or component thereof are immobilised on a solid support.

18. A test kit according to claim 17, wherein the endothelial cells are present in a confluent layer on the solid support.

19. A test kit according to claim 17 or 18, wherein the solid support comprises a polymer such as collagen, gelatin or fibronectin, or a plastic, e.g. latex, polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylalcohol, nylon, polyvinylacetate, or any suitable copolymer thereof.

20. A test kit according to claim 16, wherein the component of endoth lial cells is a surface membrane fraction or molecule.

21. A test kit according to claim 20, wherein the surface membrane molecule is a HBP receptor.

22. A test kit according to claim 15, wherein the HBP or the cell producing HBP is immobilised on a solid support.

23. A test kit according to any of claims 15-22, wherein the HBP is provided with a label.

24. A test kit according to any of claims 15-22, which further comprises, in a separate container, a labelled antibody reactive with HBP.

25. A test kit according to any of claims 15-22, which further comprises, in separate containers, a first antibody reactive with HBP and a labelled second antibody reactive with the first antibody.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00270

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: G 01 N 33/566, 33/68, C 12 Q 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	G 01 N; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	MICROVASCULAR RESEARCH, Vol. 42, 1991 G.E. Besner et al.: "Macrophages Secrete a Heparin-Binding Inhibitor of Endothelial Cell Growth", pp. 187-197, see fig. 3 and page 190, last paragraph - page 192	2,4-6, 15-19
Y	--	1-6,11, 15-19
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 265, No. 35, December 1990 B.A. Konkle et al.: "Heparin-binding Growth Factor-1 Modulation of Plasminogen Activator Inhibitor-1 Expression", pp. 21867-21873, see pages 21867-21868	1-6,11, 15-19
Y	--	
Y	WO, A1, 8908666 (NORDISK GENTOFTE A/S) 21 September 1989, see pages 1-11, 20-22 and example 6	1-6,11, 15-19
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* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th December 1992	18 -12- 1992
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Thrombosis and Haem stasis, Vol. 60, N : 2, 1988 M. Laurell et al.: "Monoclonal Antibodies Against the Heparin-Dependent Protein C Inhibitor Suitable for Inhibitor Purification and Assay of Inhibitor Complexes", see page 334 - page 339	1-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 92/00270

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 30/10/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8908666	89-09-21	AU-B-	617795	91-12-05
		AU-D-	3346289	89-10-05
		EP-A-	0409858	91-01-30
		JP-T-	3504919	91-10-31

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